NEEDLE & ROSENBERG

IMPORTANT FAX DOCUMENT

NEEDLE & ROSENBERG PC
SUITE 1000
999 PEACHTREE STREET
ATLANTA, GEORGIA 30309-3915
678-420-9300
NEEDLEROSENBERG.COM

| | |
|---|---|
| DATE | July 6, 2004 |
| NAME | Examiner Nichol |
| COMPANY | U.S. Patent and Trademark Office |
| FAX NUMBER | 571-273-0835 |
| FROM | Bruce H. Becker, M.D. |
| REFERENCE NO. | 07038.0003U2 |
| OUR FAX NUMBER | 678-420-9301 |
| NUMBER OF PAGES | 9 |

In re Application of
Leskovar, P.
U.S. Appln. No. 10/087,252
Title: "DRUGS AND METHODS FOR TREATING CANCER"

Dear Examiner Nichol:

Attached is the original unsigned Declaration of Inventor Peter Leskovar.

Unsigned COPY (signed copy is present in EDAN)

**ATTORNEY DOCKET NO. 07038.0003U2**
**PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | |
|---|---|---|
| In re Application of | ) | |
| | ) | |
| Leskovar, P. | ) | Group Art Unit: Unassigned |
| | ) | |
| Serial No. Unassigned | ) | Examiner: Unassigned |
| | ) | |
| Filed: Unassigned | ) | |
| | ) | |
| Continuation of Serial No. 09/302,642 | ) | |
| Filed: May 3, 1999 | ) | |
| | ) | |
| For: "DRUGS AND METHODS FOR TREATING CANCER" | ) | |

**DECLARATION OF PETER LESKOVAR UNDER 37 C.F.R. § 1.132**

I, Peter Leskovar, a citizen of Germany, residing at D-83026 Rosenheim, Tizianstr.11, Germany, declare that:

I am the inventor of the invention embodied in the above-referenced application, and that I have read and understand the application. The following is a description of experiments conducted by me or at my direction in accordance with the present invention which provide evidence of the efficacy and enablement of the present invention.

Animal Experiments

To our understanding, there is no chance for a high-efficiency immunostimulation by any

biological response modifiers (BRMs) as long as the inevitably costimulated suppressor cells are not depleted. In an animal model (murine B16 melanoma model), we tested the combination of a tumor-specific immunostimulation with the depletion of potential suppressor cells.

To generate a tumor-specific immunostimulation, we injected donor mice with a mixture of tumor cells plus *B. subtilis* lyophilisate. This pretreatment of donor mice resulted in the generation of tumor-specific cytotoxic T cells (CTLs) within 14 days. These tumoricidal CTLs were, however, accompanied by the costimulated suppressor cells.

To eliminate suppressor cells or their (co)inducers, we treated both the tumor-bearing mice (recipients, "R") and the donor mice (donors, "D") either by pan-T-specific or subset-specific monoclonal antibodies (Mabs) and their combinations.

In the experimental series S (S1....S6) the donor splenocytes were treated *ex vivo* by subset-specific Mabs and the recipient mice by the pan-T-specific Mab *in vivo*. In the experimental series N (N1....N6), both the donor splenocytes and recipients were treated by the same subset-specific Mabs.

The CD4 positive T cells ("helper/inducer T cells") can be considered as promoters of the CD8 positive effector suppressor cells. The so called "inducer" and "inducer/transducer" suppressor cells belong to the T4 subset and seem to have some elements in common with the more recently discussed immunosuppressive Th 2 subset. The CD8 positive T cells ("cytotoxic/suppressor T cells") include the so called "effector" suppressor cells.

The idea behind the depletion of macrophages (alone or in combination with the T4 or T8 subset) was the (co)depletion of "suppressor monocytes" which are able to induce the suppressor T cells (via Th2 cells).

The tumor specific immune stimulation in recipient mice was generated by injecting splenocytes from donor mice which received a mixture of isolated melanoma tumor cells and *B. subtilis* lyophilisate. The use of donor splenocytes is analogous to the use of preactivated lymphocytes because syngeneic mice were used and because splenocytes of mice are similar to or at least comparable to lymphocytes. Splenocytes of syngeneic mice were used because it is too difficult to withdraw blood from mice and reinject it because mice do not have a high volume of blood. *B. subtilis* is used as an activator or immunostimulator.

Note: Because the suppressor-selective Mabs are not available, the suppressor-subsets could only be co-depleted along with other non-suppressor subpopulations.

Table 1: Survival data, expressed in the number of mice

| | Ref1 | Ref2 | Ref3 | S1 | S2 | S3 | S4 | S5 | N1 | N2 | N3 | N4 | N5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| d8 | 13/13 | 22/24 | 3/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 |
| d16 | 11/13 | 17/24 | 2/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 | 6/6 |
| d32 | 0/13 | 2/24 | 1/6 | 4/6 | 3/6 | 4/6 | 5/6 | 2/6 | 4/6 | 4/6 | 2/6 | 6/6 | 2/6 |
| d48 | 0/13 | 0/24 | 0/6 | 2/6 | 1/6 | 2/6 | 2/6 | 2/6 | 2/6 | 1/6 | 1/6 | 4/6 | 0/6 |
| d64 | 0/13 | 0/24 | 0/6 | 2/6 | 1/6 | 2/6 | 1/6 | 2/6 | 2/6 | 1/6 | 1/6 | 2/6 | 0/6 |

Note: The first number stands for living mice, the second number for the total number of mice/subgroup.

d8...d64 (day8...day64): indicates how many days after injection of donor effector cells as tumor-specific immunostimulators (Ref3; S1-S5; N1-N5) or how many days after tumor excision (Ref1) and Ts elimination (Ref2), respectively. For example, d8 means 8 days after....

Ref1...only tumor excision

Ref2...tumor excision plus suppressor cell elimination (in recipient)

Ref3...tumor excision plus tumor-specific immunostimulation

S1-S5 and N1-N5...tumor excision plus suppressor cell elimination plus tumor-specific immunostimulation

Table 2: Survival data (in %)

| | Ref1 | Ref2 | Ref3 | S1 | S2 | S3 | S4 | S5 | N1 | N2 | N3 | N4 | N5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| d8 | 100 | 91,7 | 50 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| d16 | 84,8 | 70,8 | 33,3 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| d32 | 0 | 8,3 | 16,7 | 66,7 | 50 | 66,7 | 83,3 | 33,3 | 66,7 | 66,7 | 33,3 | 100 | 33,3 |
| d48 | 0 | 0 | 0 | 33,3 | 16,7 | 33,3 | 33,3 | 33,3 | 33,3 | 16,7 | 16,7 | 66,7 | 0 |
| d64 | 0 | 0 | 0 | 33,3 | 16,7 | 33,3 | 16,7 | 33,3 | 33,3 | 16,7 | 16,7 | 33,3 | 0 |

Note: Table 2 expresses survival data of the animals in terms of % of the group surviving.

Technical details

Part I: Use of anti-pan T = anti-Thy1.2-Mab in recipient mice and subpopulation-specific Mabs for donor mice splenocytes

Donor mice: Mice are inoculated with the mixture of $2x10^6$ living B16-tumor cells plus *B. subtilis*

lyophilisate (Sigma B4006). The mixture (B16-cells plus *B. subtilis*) has to be suspended in 0.05 ml of sterile PBS (or Hanks or RPMI 1640). If *B. subtilis* lyophilisate is not available in sufficient amounts, alternative bacterial lyophilisates (acetone powders) can be taken instead of the *B. subtilis* lyophilisate.

On day +14 after tumor inoculation, the spleens of these donors are removed. The removed splenocytes are treated *in vitro* by monoclonal antibodies (Mabs) to remove selectively the corresponding immunocyte subpopulation. This process thus removes suppressor cells induced in the donor so that when the donor splenocytes are injected into the recipient, no contaminating suppressor cells are introduced into the recipient after the recipient has already been treated to eliminate its own tumor suppressor cells.

In order to simplify the procedure, these subpopulations are only opsonized (not depleted) *in vitro*; their elimination occurs *in vivo* after the injection of (preopsonized) donor splenocytes into the recipients. This simplified procedure consists of the following steps: The anti-CD4 (CLO12A), the anti-CD8 (CL169) and the anti-B cells (MCA450)-Mabs have to be pre-diluted 1:10, the anti-monocyte-Mab (MCA519) 1:2 with sterile PBS (or Hanks or RPMI 1640), aliquoted for single mouse injection (0.200 ml) or for 6 mice-groups (1.200 ml) or for larger groups of daily injected mice (n x 0.200 ml) and thereafter frozen (at –20°C or –70°C) or kept at 0-4°C under sterile conditions. In addition, the donor splenocytes are centrifuged (300 g, 5 min), resuspended in 0.200 ml of the above-mentioned Mab-solution, i.e., the 1:10 or 1:2 Mab-predilutions, incubated at 37°C for 30 min and thereafter injected intravenously into the recipient mice.

Note: If combinations of 2 Mabs are used, 0.200 ml plus 0.200 ml, not 0.100 ml plus 0.100 ml

of each Mab should be used.

Recipient mice: Mice are inoculated with $2x10^6$ B16 tumor cells. On day +9 after tumor inoculation, the animals are injected with cyclophosphamide (120 mg/kg = 3 mg/25 g), and 1-3 hours before the tumor excision (on day +10 post-inoculation), all recipients are injected with anti-Thy 1.2 in exactly the same way and dose as practiced in other experiments.

S1 Use of anti CD4 (CLO12A)-Mab for the treatment of donor splenocytes

S2 Use of anti CD8 (CL169)-Mab for the treatment of donor splenocytes

S3 Use of anti-macrophage (MCA519)-Mab for the donor splenocytes

S4 Use of anti CD4 plus anti-macrophage-Mabs for the donor splenocytes

S5 Use of anti CD8 plus anti-macrophage-Mabs for the donor splenocytes

Note: All recipients were treated *in vivo* by the pan-T-specific anti-Thy1.2-Mab.

Part II: Use of the same subpopulation-specific Mabs, both for donor splenocytes and in recipient mice

Comment: The only difference between the experiments of the "Part I" and those of the "Part II" is the replacement of the pan-T-specific, i.e., anti-Thy1.2-Mabs, in the "Part I" experiments by the subpopulation-specific Mabs in the "Part II" experiments. Therefore, the recipients of the type (R1) and (R2) are different in "Part I" and "Part II", whereas the donors (D1) and (D2) are identical in both experimental series.

Note: The dose of injected subpopulation-specific Mabs: 0.300 ml of the 1:10 (or 1:2) prediluted Mab.

Donor mice: Identical with (D1) and (D2) donors from "Part I." The mice of D1 and the mice of D2 were the same kind of mice, but the animals used in D1 were different from the animals used in D2.

Recipient mice: Both recipient types (R1) and (R2) differ only in the replacement of anti-Thy.1.2-Mab (Part I) by subpopulation-specific Mabs (Part II), such as anti CD4-, anti CD8-, anti-macrophage, anti CD4- plus anti-macrophage, and anti CD8- plus anti-macrophage-Mabs.

N1 Use of anti CD4 (CLO12A)-Mab for donor mice (*in vitro*) and the same anti CD4 (CLO12A)-Mab for recipient mice (*in viva*)

N2 Use of anti CD8 (CL169)-Mab for donor mice (*in vitro*) and of the same anti CD8 (CL169)-Mab for recipient mice (*in vivo*)

N3 Use of anti-macrophage (MCA519)-Mab for donor mice (*in vitro*) and the same Mab for recipient mice (*in vivo*)

N4 Use of anti CD4 plus anti-macrophage-Mabs for donor mice (*in vitro*) and of the same Mabs for recipient mice (*in vivo*)

N5 Use of anti CD8 plus anti-macrophage-Mabs for donor mice (*in vitro*) and for recipient mice (*in vivo*)

In the present experiments, using the methods claimed, a significant improvement in the survival of the treated mice was observed. Thus, there is a very strong basis to believe that the methods as described herein and claimed will be effective to treat cancer.

**ATTORNEY DOCKET NO. 07038.0003U2**
**Serial No. Unassigned**

I declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or document or any patent issuing therefrom.

Date

Peter Leskovar